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PRINCIPAL INVESTIGATOR: Denise C. Connolly, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111-2412

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Denise C. Connolly, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111-2412

E-Mail: Denise.connolly@fccc.edu

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About one out of every ten cases of epithelial ovarian cancer is inherited. Unlike non-hereditary (sporadic) ovarian cancer, some of the underlying genetic causes of hereditary ovarian cancer are well understood. The majority, >90%, of inherited cases are the result of inherited mutations in the breast cancer associated gene 1 (BRCA1). In addition to mutations of BRCA1, mutations of the p53 gene are often found in patients with breast and ovarian cancer syndrome. Based on the importance of both of these genes in the development of this type of ovarian cancer, we hypothesize that inactivation of BRCA1 and p53 in the ovaries of mice will result in epithelial ovarian cancer in the animals. We have obtained mouse strains that conditionally express the BRCA1 and p53 genes. Tissue-restricted exposure to cre recombinase results in excision of LoxP flanked (floxed) sequences in these mice and expression of the mutated forms of BRCA1 and p53. To achieve cre recombinase expression that is restricted to ovary we have employed localized delivery of Adenovirus-Cre by intrabursal injection of virus. We also developed transgenic mice that should express cre recombinase in a reproductive tissue-restricted and drug inducible manner.

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MODELING HUMAN EPITHELIAL OVARIAN CANCER IN MICE BY ALTERATION OF EXPRESSION OF THE *BRCA1* AND/OR *P53* GENES

Introduction:

About one out of every ten cases of epithelial ovarian cancer is inherited. Unlike non-hereditary (sporadic) ovarian cancer, some of the underlying genetic causes of hereditary ovarian cancer are well understood. The majority, >90%, of inherited cases are the result of inherited mutations in the breast cancer associated gene 1 (*BRCA1*). This gene was originally identified based on genetic linkage to families with an increased risk of developing breast and ovarian cancer. It is involved in controlling normal cellular growth and is thought to suppress the growth of tumors. That is, if BRCA1 is mutated, the risk to develop breast and ovarian cancer increases. Another gene that is important in the development of cancer is the *p53* gene. It also helps maintain normal cellular growth and is the most commonly mutated gene in all human cancers. It has been shown to be mutated in at least 50% of all cases of epithelial ovarian cancer. In addition to mutations of *BRCA1*, mutations of the *p53* gene are often found in patients with breast and ovarian cancer syndrome. Based on the importance of both of these genes in the development of this type of ovarian cancer, we hypothesize that inactivation of *BRCA1* and *p53* in the ovaries of mice will result in epithelial ovarian cancer in the animals.

The objectives of this funded proposal are to:

1. develop mouse models of human epithelial ovarian cancer by inactivation of BRCA1 and p53 singly or at the same time in the mouse ovarian surface epithelial cells;
2. investigate whether there is a difference between the complete absence of p53 or the presence of a dominantly acting p53 mutant in ovarian tumorigenesis in mice; and,
3. identify genes and cellular pathways, downstream of BRCA1 and p53 inactivation/mutation, that contribute to ovarian carcinogenesis.

Body:

The approved Statement of Work for this proposal consisted of seven tasks for the 36 month funding period. Specific tasks that were proposed for the first 12 months of the funding period are included in tasks 1-4 and accomplishments, task by task, are reviewed below. Tasks outlined in original Statement of Work appear in italics. Work accomplished listed below each task in regular font. Tasks 5-7 include work proposed for the second and third years of the project; therefore work related to these tasks has not yet been initiated and does not pertain to this review.

Task 1. Obtain mice with conditional BRCA1 and/or p53 alleles (months 1-9).

a. Obtain breeding pairs of BRCA1 floxed and p53 floxed mice from the Mouse Models of Human Cancer Consortium (http://mouse.ncifcrf.gov).

b. Establish breeding colonies of each strain ($BRCA1^{loxP/loxP}$ and $p53^{loxP/loxP}$).

c. Mouse genotyping analysis by PCR and Southern blotting.

d. Cross $BRCA1^{loxP/loxP}$ and $p53^{loxP/loxP}$ mice to establish a breeding colony of $BRCA1^{loxP/loxP}/p53^{loxP/loxP}$ mice.

e. Genotyping analysis of $BRCA1^{loxP/loxP}/p53^{loxP/loxP}$ mice.

f. *Approximately 100 mice will be required for each conditional strain to obtain 36-40 age matched females for subsequent experiments.*

Mating pairs of *BRCA1*$^{loxP/loxP}$ and *p53*$^{loxP/loxP}$ mice were obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancer Consortium (MMHCC) Mouse Repository in November, 2003. Breeding colonies for each strain were established and are currently being maintained. As the mice we received from the MMHCC Mouse Repository were homozygous mating pairs, all resulting offspring are homozygous. Protocols for genotyping the *BRCA1*$^{loxP/loxP}$ and *p53*$^{loxP/loxP}$ mice were obtained from the laboratory of Dr. Anton Berns, where each of these strains were initially developed. By PCR amplification of genomic DNA, we verified that both the *BRCA1*$^{loxP/loxP}$ and *p53*$^{loxP/loxP}$ colonies were indeed homozygous for the LoxP flanked (floxed) alleles of *BRCA1* and *p53* respectively. The *p53*$^{loxP/loxP}$ mice are not particularly robust breeders which delayed the ability to build a sufficiently large colony of these mice. To obtain *BRCA1*$^{loxP/loxP}$/*p53*$^{loxP/loxP}$ mice that express both floxed *BRCA1* and *p53*, matings of *BRCA1*$^{loxP/loxP}$ X *p53*$^{loxP/loxP}$ mice were initiated. The same protocols for genotyping both *BRCA1* and *p53* mice were used to evaluate the status of each allele in the offspring. From the time these crosses were initiated in June 2004, it took approximately six months to begin to obtain offspring that were homozygous for both floxed alleles (*BRCA1*$^{loxP/loxP}$/*p53*$^{loxP/loxP}$). At this time, Task 1 is complete and we are maintaining colonies of *BRCA1*$^{loxP/loxP}$, *p53*$^{loxP/loxP}$ and *BRCA1*$^{loxP/loxP}$/*p53*$^{loxP/loxP}$ mice for subsequent experiments.

Task 2. Generate, characterize, and establish breeding colonies of TgMISIIR-rtTA-Cre transgenic mice (months 1-15).

a. Purify transgene construct from plasmid pMISIIR-rtTA-Cre.

b. Generate transgenic TgMISIIR-rtTA-Cre mice (injection of embryos, implantation gestation).

c. Genotyping analysis of founder animals by PCR and Southern blot analysis.

d. Breed founders to generate F1 mice.

e. Genotyping analysis of F1 offspring.

f. Establish breeding colonies of five individual TgMISIIR-rtTA-Cre transgenic lines of mice by crossing F1 offspring from each line.

g. Characterize offspring of individual TgMISIIR-rtTA-Cre transgenic lines for expression of the Cre recombinase transgene following induction with systemic doxycycline by:

- *Immunohistochemical detection of Cre recombinase in mouse tissues.*
- *Crosses of transgenic offspring with Rosa26R*^{tm1Sor} *reporter mice (mice) and detection of β-Galactosidase expression in mouse tissues.*

h. Establishment and characterization of 5 transgenic lines requires a total of approximately 80 mice.

We obtained the Core Construct plasmid (depicted schematically in Figure 1) from Dr. Alex Nikitin at Cornell University, Ithaca, NY. Briefly, this plasmid contains the reverse tetracycline trans-activator (rtTA) and the Cre-recombinase (Cre) genes separated by a p53 intron. The rtTA is adjacent to a multiple cloning sequence into which a gene promoter of choice can be cloned to direct transcription of the rtTA gene. The Cre gene is under the transcriptional control of a tetracycline (tet) responsive minimal operator (Tet-O) sequence. A 1.2 kb fragment or he 5' upstream regulatory sequence of the Müllerian inhibitory substance type II receptor (MISIIR) gene was subcloned into the Core Construct to create the pMISIIR-rtTA-Cre transgenic plasmid construct in which transcriptional control of the rtTA gene is mediate by this promoter element.

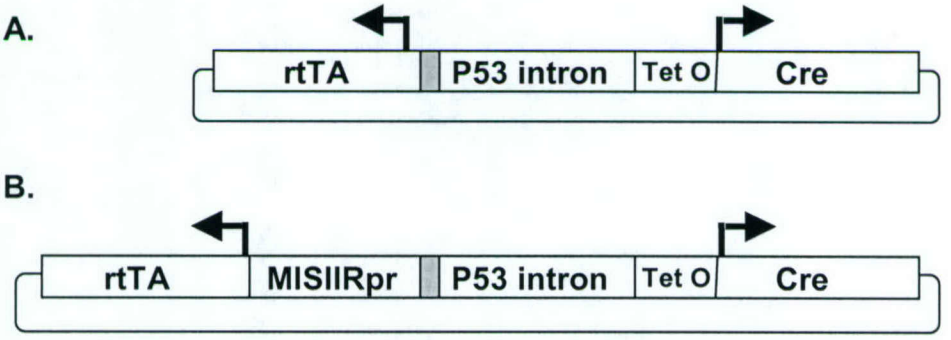

Figure 1. A) Schematic diagram of Core Construct consisting of the rtTA and the Cre recombinase genes separated by a p53 intron. Transcriptional orientation is indicated by the arrows. The grey shaded area between the rtTA and p53 is a multiple cloning sequence (MCS). (Diagram is not to scale). B) Schematic diagram of MISIIR-rtTA-Cre plasmid. An SpeI/XbaI digested DNA fragment containing the 1.2 kb MISIIR gene promoter element was subcloned in to the Core construct at the NheI site in the MCS.

The 6.2 kb MISIIR-rtTA-Cre transgene fragment was removed from the pMISIIR-rtTA-Cre plasmid by digestion with the restriction endonuclease I-SceI. The transgene fragment was gel purified and prepared for injection of fertilized embryos as described (1). Pups were genotyped by PCR amplification of purified tail DNA using primers for both the rtTA and Cre genes. We obtained ten Tg*MISIIR-rtTA-Cre* transgenic founders of which seven founders resulted in the establishment of transgenic lines that transmit the transgene to offspring. Offspring of these seven transgenic lines were crossed to Rosa26R^{tm1Sor} reporter mice. Offspring of these crosses were genotyped for rtTA and Cre genes and for the floxed allele of the β-galactosidase (β-gal) gene. Female mice that contained the Tg*MISIIR-rtTA-Cre* transgene and the floxed β-gal allele were treated with Doxycycline (Dox) by administration of 2.0 mg/ml Dox to the drinking water for a period of two weeks to induce transcription of the Cre recombinase gene. These animals were recently sacrificed to determine whether Cre mediated excision of the floxed LacZ reporter gene in these mice occurs in a tissue restricted and Dox-inducible manner. We also sacrificed mice that did not receive Dox in the drinking water to ensure that the induction of the Cre recombinase is tightly regulated. Reproductive organs (ovary, Fallopian tube and uterus) as well as other normal tissues and organs were collected and snap frozen. These specimens will be evaluated for Cre-recombinase mediated excision of floxed sequences and expression of the β-gal allele by β-galactosidase staining of frozen tissue sections by a standard protocol (described in detail below). Results of these experiments are pending at the time of this submission. Most of task 2 is complete. We are awaiting final results.

Task 3. Adenovirus-Cre mediated inactivation loxP flanked (floxed) alleles of $BRCA1^{loxP/loxP}$, $p53^{loxP/loxP}$ *and* $BRCA1^{loxP/loxP}/p53^{loxP/loxP}$ *mice (months 6-18)*

a. *Obtain sufficient numbers of animals that are homozygous for the floxed allele. Initially, 36 animals will be required for each strain evaluated.*
b. *Perform intrabursal injections on 30 female mice with recombinant adenovirus-Cre (AdCMVCre) or 6 female mice with phosphate buffered saline as controls.*
c. *Monitor and evaluate animals for tumor formation, frequency and latency.*
d. *Histopathological evaluation of tissues of mice from each test group.*

Conditional inactivation of the floxed alleles in $BRCA1^{loxP/loxP}$, $p53^{loxP/loxP}$ and $BRCA1^{loxP/loxP}/p53^{loxP/loxP}$ mice requires the presence of Cre recombinase. One approach for restricted excision of floxed sequences in the ovarian surface epithelium is intrabursal injection of Ad-Cre. As this has been previously shown to be an effective approach (2, 3), we have decided to pursue this method. Intrabursal injection requires significant technical expertise and survival surgery on mice. In order to demonstrate our capability to successfully perform these injections, we performed short term pilot experiments in which female mice received intrabursal injections of Adenovirus LacZ. Briefly, to synchronize ovulation, female mice were injected intraperitoneally with 5 U of pregnant mare serum gonadotropin, followed 48 hours later by injection with 5 U of human chorionic gonadotropin (hormones purchased from Sigma, St. Louis, MO). 1.5 days following the last hormone injection, mice were given a single intrabursal injection of Ad5-CMV-ntLacZ virus (Gene Transfer Vector Core, University of Iowa, Iowa City, IA) by inserting a needle into the oviduct near the infundibulum into the ovarian bursa. Virus was diluted into sterile PBS to deliver $5x10^7$ pfu of virus per 10 μl intrabursal injection. For short-term analysis of LacZ expression, β-galactosidase staining was performed. Mice were sacrificed 3-4 days after Ad5-CMV-ntLacZ injection. Ovaries were removed and frozen in OCT medium (Tissue-Tek), then 10 μm sections cut with a cryostat. Sections were fixed for 10 minutes in 0.5% glutaraldehyde, followed by two rinses with PBS+0.1% Tween-20, then stained with X-gal solution (1 mg/ml X-gal (Promega, Madison, WI), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in phosphate-buffered saline (PBS)) at 37°C overnight in a humidified chamber. Sections were rinsed twice in PBS+0.1% Tween-20, counterstained with Nuclear Fast Red, dehydrated with alcohols, cleared in xylenes, and coverslipped using Permount (Fisher). Results of the β-galactosidase staining are depicted in Figure 2 and show intrabursal injection of Ad-CMV-ntLacZ resulted in infection that was confined to the single layer ovarian surface epithelial cells that cover the ovary.

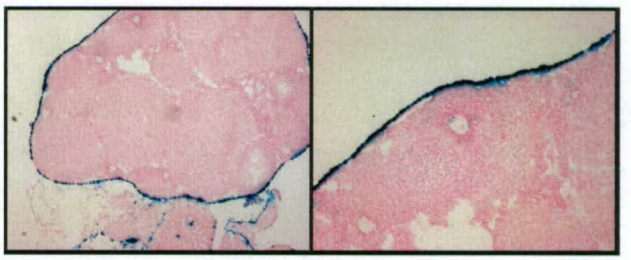

Figure 2. β-galactosidase staining of ovaries from wild type mouse that received intrabursal injection of Ad-LacZ. (Magnification: Left, 4x and right, 10x)

Based on the demonstration of successful use of the technique for intrabursal injection of adenovirus, we initiated intrabursal injections of $BRCA1^{loxP/loxP}$ mice with Ad-CMVCre (Gene Transfer Vector Core, University of Iowa, Iowa City, IA). Methods for synchronization of ovulation and virus injection were as described for Ad-LacZ. At the time of submission of this progress report, 21 female $BRCA1^{loxP/loxP}$ mice between 6-8 weeks, 5 $BRCA1^{loxP/loxP}$ mice at 11

weeks and 5 *BRCA1*$^{loxP/loxP}$ mice at 14 weeks received intrabursal injections of Ad-CMVCre. We performed short-term analysis of excision of floxed sequences by PCR amplification of the *BRCA1* allele. Briefly, one of the 14 week old mice was sacrificed one week post-injection with Ad-CMVCre and one ovary was removed and incubated in lysis buffer (0.05 M Tris HCl, 0.01 M EDTA, 0.1 M NaCl, 0.1% SDS) containing 1.5 mg/ml proteinase K at 55°C for 30 minutes to digest the surface epithelial layer of the ovary. At the end of the 30 minute digestion, the ovary was removed into a fresh aliquot of buffer containing proteinase K and digested overnight at 55°C. All other tissues (liver, uterus) were digested overnight at 55°C. The 5' loxP and 3' loxP sites in *BRCA1*$^{loxP/loxP}$ mouse were amplified using the intron 4 an intron 13 specific primer pairs, int4-fwd 5'-TATCACCACTGAATCTCTACCG and int4-rev 5'-GACCTCAAACTCTGAGATCCAC and int13-fwd 5'-TATTCTTACTTCGTGGCACATC and int13-rev 5'-TCCATAGCATCTCCTTCTAAAC, respectively. The PCR products for intron 4 are: wild type = 391 nt and floxed = 461 nt; and for intron 13 are: wild type = 492 nt and floxed = 562 nt. As expected, only the floxed *BRCA1* allele (loxP sites in both introns 4 and 13) was detected in liver, uterus and ovary of the *BRCA1*$^{loxP/loxP}$ mouse (Fig.3A, lanes 1, 3, 4, and 5). Only the wild type allele was detected in the wild type mouse (Fig. 3A, lane 2) and in a *BRCA1*$^{WT/loxP}$;CMV-Cre mouse that has constitutive expression of cre-recombinase and the floxed sequences are excised (Fig. 3A, lane 6). Both the wild type and floxed alleles were present in the heterozygous *BRCA1*$^{WT/loxP}$ mouse (Fig. 3A, lane 7). Based on previously reported results (2, 3) and our results shown above, where intrabursal injection of adenovirus results in infection of only the ovarian surface epithelial (OSE) cells, we expect cre-mediated excision of the *BRCA1*$^{loxP/loxP}$ allele to likewise occur in the OSE cells. To detect cre-mediated excision of floxed sequences in the ovary of a mouse that received intrabursal injection of Ad-CMVCre, the ovary was digested for a short period of time (30 min.) to digest only the outer surface of the ovary (containing the OSE cells) and avoid complete digestion of the ovary which could result in the presence of such an excess of DNA that the excised product would be undetectable. After the initial digestion, the ovary was placed in a fresh aliquot of lysis buffer and allowed to continue digestion overnight. To detect the recombined allele, we used primers int4-fwd and int13-rev which are expected to result in PCR amplification and detection of a 431 nt product only in tissues where cre-mediated excision occurred.

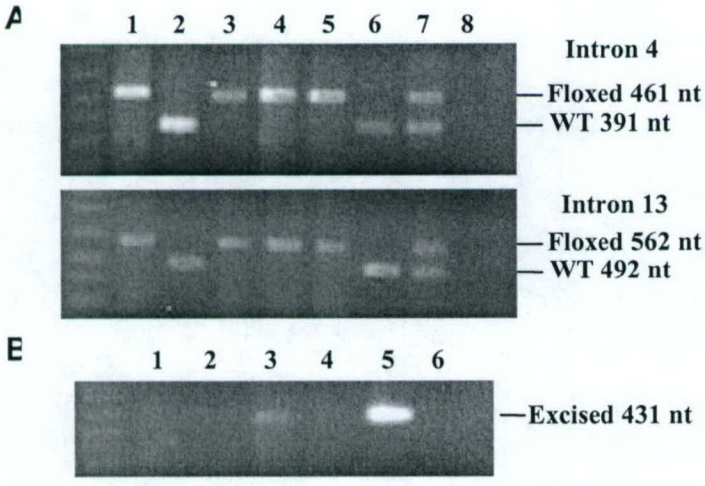

Figure 3. A) PCR amplification of LoxP containing sites in intron 4 (top) and intron 13 (bottom). The LoxP flanked *BRCA1* intron 4 PCR product is 461 nt and the wild type intron 4 PCR product is 391 nt. Lane 1- liver *BRCA1*$^{loxP/loxP}$ mouse, Lane 2, wild type mouse, Lanes 3-5, *BRCA1*$^{loxP/loxP}$ mouse (3- uterus, 4- ovary digested 30 min., 5- 2nd digest of ovary [12 h]), Lane 6, *BRCA1*$^{WT/loxP}$;CMV-Cre mouse, Lane 7-*BRCA1*$^{WT/loxP}$ mouse and Lane 8- H_2O control. **B)** PCR amplification of excised *BRCA1* alllele. The excised *BRCA1*$^{loxP/loxP}$ allele is detected at 431 nt and present only in tissues exposed to Cre recombinase. Lanes 1-4, *BRCA1*$^{loxP/loxP}$ mouse (1- liver, 2- uterus, 3- ovary digested 30 min. and 4- 2nd digestion of ovary for 12 h.), Lane 5- *BRCA1*$^{WT/loxP}$;CMV-Cre mouse and Lane 6- H_2O control.

Results are depicted in Figure 3B, where the excised product is detectable in DNA isolated from the first short digestion of the ovary (Lane 3), but not in DNA isolated from the liver, uterus or

second digestion of the ovary (lanes 1, 2, and 4) from the Ad5-CMVCre injected *BRCA1*$^{loxP/loxP}$ mouse. Lane 5 of Figure 3B depicts a positive control for excision, showing the PCR product from *BRCA1* allele present in a *BRCA1*$^{WT/loxP}$;CMV-cre mouse in which cre is constitutively expressed and the floxed sequence is excised.

As previously stated, he *p53*$^{loxP/loxP}$ mice are poor breeders, therefore all of the female *p53*$^{loxP/loxP}$ mice bred until to this point have been used to establish the *BRCA1*$^{loxP/loxP}$;*p53*$^{loxP/loxP}$ mice. Now that the colony has been established subsequent female *p53*$^{loxP/loxP}$ and *BRCA1*$^{loxP/loxP}$;*p53*$^{loxP/loxP}$ mice not required for colony maintenance will receive intrabursal injection of Ad5-CMVCre.

Task 4. Obtain and establish breeding colonies of dominant-negative p53 (Tgp53R172HΔg) mice (months 8-15).

a. *Obtain breeding pair of p53R172HΔg mice from Dr. Guillermina Lozano's laboratory (MD Anderson, Houston, TX).*
b. *Establish breeding colony of these mice.*
c. *Genotyping analysis by PCR and Southern blot analysis.*
d. *Approximately 160 mice will need to be bred to obtain 36-40 female mice that are heterozygous for the mutated p53 allele.*

A breeding pair of p53R172HΔg mice were obtained from Dr. Guillermina Lozano's laboratory (MD Anderson, Houston, TX) March 25, 2004. The mice were rederived in the Laboratory Animal Facility at Fox Chase Cancer Center. The rederived mice were obtained at the end of June 2004. We are successfully maintaining a colony of these mice and have started to breed them to the *BRCA1*$^{loxP/loxP}$ mice. Offspring of these crosses have been obtained and genotypes confirmed by PCR analysis.

All mouse procedures described in this progress report were approved by Fox Chase Institutional Animal Care and Use Committee.

Key Research Accomplishments:

- Establishment of breeding colony of *BRCA1*$^{loxP/loxP}$/*p53*$^{loxP/loxP}$ mice
- Establishment of seven stable transgenic lines of Tg*MISIIR-rtTA-Cre* transgenic mice
- Crosses of Tg*MISIIR-rtTA-Cre* transgenic mice with Rosa26R^{tm1Sor} reporter mice to establish Tg*MISIIR-rtTA-Cre*;*Rosa26R*^{tm1Sor} mice for functional characterization of tissue restricted drug-inducible expression of the Tg*MISIIR-rtTA-Cre* transgenic mice
- Pilot experiments indicating successful intrabursal injection of Adenovirus
- Intrabursal injection of 21 *BRCA1*$^{loxP/loxP}$ mice Adenovirus-Cre recombinase (Ad-Cre)
- Demonstration of cre-mediated excision of floxed *BRCA1* sequences in the ovary of a mouse that received intrabursal injection of Ad-cre

Reportable Outcomes:

As we are only one year into the funding period, there are no reportable outcomes at the time of submission of this progress report.

Conclusions:

Based on the approved statement of work and the results obtained to date, this project is on track and at the expected stage.

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3. **Flesken-Nikitin, A., K. C. Choi, J. P. Eng, E. N. Shmidt, and A. Y. Nikitin. 2003. Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res 63:3459-63.**

Appendices:

None